

Activation of decarboxyfactor X by a protein from Russel's Viper venom

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ACTIVATION OF DECARBOXYFACTOR X BY A PROTEIN FROM RUSSELL'S VIPER VENOM

PURIFICATION AND PARTIAL CHARACTERIZATION OF ACTIVATED DECARBOXYFACTOR X

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Summary

1. Incubation of decarboxyfactor X with the factor X-activating enzyme from Russell's Viper venom revealed the generation of amidase activity towards Bz-Ile-Glu-Gly-Arg-pNA, but not of activity in blood coagulation.

2. The rate of activation of both factor X and decarboxyfactor X depends on the ability of the zymogens to bind Ca^{2+} . The relationship between Ca^{2+} concentration and velocity of the activation reaction is sigmoid in the case of factor X, but hyperbolic with decarboxyfactor X.

3. Activated decarboxyfactor X was purified by powder column electrophoresis.

4. Identical changes of primary structure accompanied the activation of factor X and decarboxyfactor X. Identical molecular weight and common antigenic determinants were found in factor X_a and decarboxyfactor X_a . The amino acid composition was identical except for 12 glutamic acid residues in decarboxyfactor X_a and γ -carboxyglutamic acid residues in factor X_a .

5. Unlike factor X, activated factor X has a very low electrophoretic mobility in the presence of Ca^{2+} at pH 8.6. This is probably due to self association of factor X_a under the influence of Ca^{2+} . The electrophoretic mobility of activated decarboxyfactor X is only slightly decreased compared to decarboxyfactor X in the presence of Ca^{2+} .

Introduction

Two alternative modes of activation of factor X exist in the physiological blood coagulation process: the extrinsic and intrinsic systems. The extrinsic pathway requires tissue thromboplastin and factor VII [1–5]. Via the intrinsic

pathway factor X is activated by a product of activated factor IX, factor VIII, phospholipids and Ca^{2+} [6–11].

Several non-physiological modes of activation of factor X have also been described, utilizing Russell's Viper venom [12–16] and trypsin [17,18].

Recent studies [4,13,14] showed that the conversion of factor X (molecular weight 55 900) to factor X_a by factor IX_a and factor VIII, factor VII and tissue factor, a protease from Russell's Viper venom or pancreatic trypsin, is due to cleavage of an activation peptide from the NH_2 -terminal region of the heavy chain. This cleavage occurs between Arg-51 and Ile-52, giving rise to factor $\text{X}_{a\alpha}$ (molecular weight 47 200) and an activation peptide (molecular weight 8700). Factor $\text{X}_{a\alpha}$ is converted into factor $\text{X}_{a\beta}$ (molecular weight 44 200) by hydrolysis of a second peptide bond between Arg-290 and Gly-291 at the C-terminal region of the heavy chain. Factor $\text{X}_{a\alpha}$ and factor $\text{X}_{a\beta}$ have equivalent coagulant activity. Factor X activated by one of the agents described above is the protein that contributes the active site to prothrombinase, the physiological activator of prothrombin consisting of factor X_a , factor V, phospholipids, and Ca^{2+} [19]. Factor X_a alone has a small prothrombinase activity.

Unlike factor X, decarboxyfactor X lacks the ability to bind Ca^{2+} , and does not function normally in blood coagulation [43]. To determine whether this is because decarboxyfactor X cannot be split by factor X activating agents or because "activated" decarboxyfactor X is still inactive in prothrombinase is the purpose of this article.

Materials and Methods

All chemicals were analytical grade and obtained from Merck. Factor X and decarboxyfactor X were obtained according to the purification procedures reported earlier [20]. Crude Russell's Viper venom was obtained from Sigma Chemical Co., St. Louis, U.S.A. and the factor X activator from it was purified by the method of Schiffman [24]; it will be designated "Viper venom" in the rest of the article. The chromogenic substrate Bz-Ile-Glu-Gly-Arg-pNA (S-2222) was a kind gift of Dr. G. Claeson (A.B. Bofors Nobel Division, Peptide Research, Mölndal, Sweden). *p*-Nitrophenylguanidino-benzoate (NPGb) was purchased from Biochemical Nutrition Corp.

Amino acid and NH_2 -terminal amino acid analysis

The amino acid composition was determined by means of the method according to Spackman [25]. Aminoterminal amino acid analysis was performed according to Gray [26]. γ -Carboxyglutamic acid was determined with the method of Zytkevich [27]. More details about the analysis were reported earlier [20].

Protein concentration determination

Concentration of total protein in solutions was determined by absorbance at 280 nm, assuming $A_{1\text{cm}}^{1\%}$ for factor X = 12.4 [28]. The same value could be used for determination of decarboxyfactor X concentration because the molecular weight and amino acid composition for factor X and decarboxyfactor X are equivalent. An $A_{280\text{nm}}^{1\%}$ of 10.0 was employed for the venom coagu-

lant protein [15] and $A_{280\text{nm}}^{1\%}$ of 12.2 for factor X_a and decarboxyfactor X_a [29].

Gel electrophoresis

Analytical polyacrylamide gel electrophoresis, disc-gel electrophoresis in the presence of sodium dodecyl sulphate and agarose gel electrophoresis were performed as reported earlier [20].

Immunochemical methods

The one-dimensional crossed immunoelectrophoresis, employed for decarboxyfactor X determination using monospecific antbovine factor X antiserum, was performed using the method of Laurell [30] as reported earlier [20]. Double immunodiffusion was performed according to Ouchterlony [31].

Factor X_a assay

Factor X was activated in suitable mixtures as described under Materials and Methods. At intervals, 10- μ l aliquots were removed from the activation mixtures and diluted with 1.0 ml Michaelis buffer containing 10 mM EDTA. The samples were further diluted with Michaelis buffer depending on the degree of activation. Subsequently, an aliquot (0.1 ml) of the final dilution was incubated at 37°C for 30 s with 0.1 ml cephalin prepared from bovine brain with the method of Bell and Alton [32], and 0.1 ml factor X-deficient bovine plasma obtained as described in ref. 23. Clotting was initiated by adding 0.1 ml of 33 mM CaCl_2 solution to the incubation mixture. The clotting times were converted into units obtained by a reference curve of the logarithm of clotting time against the logarithm of dilutions of activated factor X. One unit factor X_a activity per ml was defined as the activity of 10 μ g factor X_a per ml, i.e., about the amount that can be generated in 1 ml normal plasma.

Active site titration

Titration of factor X_a and decarboxyfactor X_a with NPGB was performed according to Smith [33]. In a typical titration the sample cuvette contains 0.3 mg per ml of factor X_a or decarboxyfactor X_a in 0.1 M sodium barbital, pH 8.3, with a final volume of 300 μ l. The reference cuvette contains the same volume of buffer solution alone. To each cuvette 10 μ l of a 0.01 M solution of NPGB in dimethylformamideacetonitrile (1 : 3, v/v) was added simultaneously.

The reaction was followed at 410 nm in an Aminco DW-2 spectrophotometer at 25°C. The concentration of *p*-nitrophenol released during the reaction was calculated from the zero time intercept of the extrapolated steady state absorbance. In the Aminco DW-2 spectrophotometer A_{410} for *p*-nitrophenol at pH 8.3 was calculated as 17 500.

Amidase activity assay

The amidase activity of activated factor X and activated decarboxyfactor X was measured according to Svendsen et al. [34]. At intervals, 10- μ l aliquots were removed from the incubation mixture and diluted with 90 μ l of 0.05 M Tris · HCl/0.05 M imidazole buffer (pH 8.3) containing 0.1 M NaCl and 10 mM EDTA. To obtain a final volume of 350 μ l, 320 μ l 0.05 M Tris · HCl/0.05 M

imidazole buffer (pH 8.3) containing 0.1 M NaCl, 25 μ l solution of Bz-Ile-Glu-Gly-Arg-pNA (S-2222) in water (2 mM) and 5 μ l of the diluted sample were mixed. The initial rate of hydrolysis was measured in an Aminco DW-2 spectrophotometer, operating in the dual wavelength mode. Analysis was conducted at 37°C and amidase activity is expressed as $\Delta A_{391-344}/\text{min}$.

Rate of conversion of factor X and decarboxyfactor X

The following incubation procedure was used when factor X and decarboxyfactor X were activated by Viper venom. Buffer solution of 0.05 M Tris · HCl/0.05 M imidazole (pH 8.3), containing 0.1 M NaCl, Russell's Viper venom-X, S-2222 and factor X or decarboxyfactor X was added to a microcuvette (Helma type 105 OS with 10 mm lightpath). The final concentrations of the reaction components are given in the legend to Fig. 3. The endogenous rate of hydrolysis of S-2222 was measured before the reaction was initiated by addition of Ca^{2+} . All assays were performed in an Aminco DW-2 spectrophotometer operating in the dual wavelength mode. The hydrolysis of S-2222 was measured at 391 nm with 344 nm as the reference wavelength. The determination was carried out at 37°C and amidase activity was expressed as $A_{391-344}/\text{min}$.

Preparation of Factor X_a

A solution containing 48 mg of factor X and 120 ml of 0.025 M Tris · HCl buffer (pH 8.3), 0.2 M in NaCl was made 5 mM with respect to CaCl_2 and incubated at 37°C for 30 min with 0.8 mg of the factor X-converting protein from Russell's Viper venom. Complete activation of factor X was achieved as judged by factor X_a activity assay of the incubation mixture. The reaction mixture was then diluted twice with 0.05 M Tris · HCl (pH 7.1) containing 0.06 M trisodium citrate and dialyzed overnight against the same buffer. Then the incubation mixture was applied to a column (30 \times 1.5 cm) of DEAE-Sephadex equilibrated in the dialysis buffer. Factor X_a was eluted as a single peak by a linear NaCl gradient (0–0.6 M) in 0.05 M Tris · HCl buffer (pH 8.0) containing 0.1 M trisodium citrate.

Preparation of activated decarboxyfactor X

Semi-purified decarboxyfactor X obtained after QAE-Sephadex chromatography [20] was dialyzed against 0.025 M Tris · HCl buffer, pH 7.4, containing 0.1 M NaCl. Decarboxyfactor X, 40 mg in 25 ml of dialysis buffer, was incubated with 1 mg Russell's Viper venom in the presence of 10 mM Ca^{2+} at 37°C for 100 min. The reaction was stopped by the addition of EDTA to a final concentration of 10 mM. The reaction mixture was then dialyzed against 2 mM Tris · glycine buffer, pH 8.3, containing 1 mM EDTA. Activated decarboxyfactor X was isolated by powder column electrophoresis according to Rosenbaum [35]. All buffers contained 5 mM calcium lactate, in order to obtain a separation between decarboxyfactor X_a and residual amounts of factor X_a . The column of the LKB 7900 Uniphor apparatus was packed with a slurry of Biogel P-300 (100–200 mesh) in 0.025 M Tris · glycine buffer, pH 8.3. First, a volume of 25 ml of 0.025 M Tris · HCl buffer, pH 8.2, was allowed to flow into the column and then the sample (30 mg protein dissolved in 25 ml Tris · HCl buffer). After the sample had entered the column, Tris/glycine buffer,

pH 8.3, was placed on the top of the column. A potential difference of 600 V was applied and the power was maintained at 16 W throughout the course of the separation. The sample was eluted from the bottom of the column with Tris · glycine buffer, pH 8.3, at a flow rate of 30 ml per h. Fractions of 3 ml were collected. Decarboxyfactor X_a was determined by amidase assay as described elsewhere.

Results

Activation of bovine factor X and decarboxyfactor X by Russell's Viper venom

The generation from purified factor X of coagulant factor X_a activity and amidase activity upon action of Viper venom is shown in Fig. 1A. The weight ratio of venom to factor X in this experiment was 1 : 80. The results shows a good agreement between formation of factor X_a activity and amidase activity. The reaction is essentially complete within 3 min. It is also demonstrated that EDTA completely inhibits the activation reaction.

Fig. 1B shows the same experiment with decarboxyfactor X instead of factor X. For the activation of decarboxyfactor X the weight ratio of decarboxyfactor X to Viper venom was 50 : 1. In contrast to an almost negligible formation of factor X_a activity within 2 min, a progressive increase in amidase activity is demonstrated. The small factor X_a activity generated by Viper venom from decarboxyfactor X activity is most likely due to a contamination of factor X (less than 5%). Under the conditions used, decarboxyfactor X is almost completely activated within 150 min. As in the activation of factor X, EDTA inhibits the reaction completely. The final yield of amidase activity per mol of zymogen with decarboxyfactor X is about 90% of that obtained with factor X.

Molecular weight changes in factor X and decarboxyfactor X upon activation were monitored by sodium dodecyl sulphate (SDS) gel electrophoresis. With

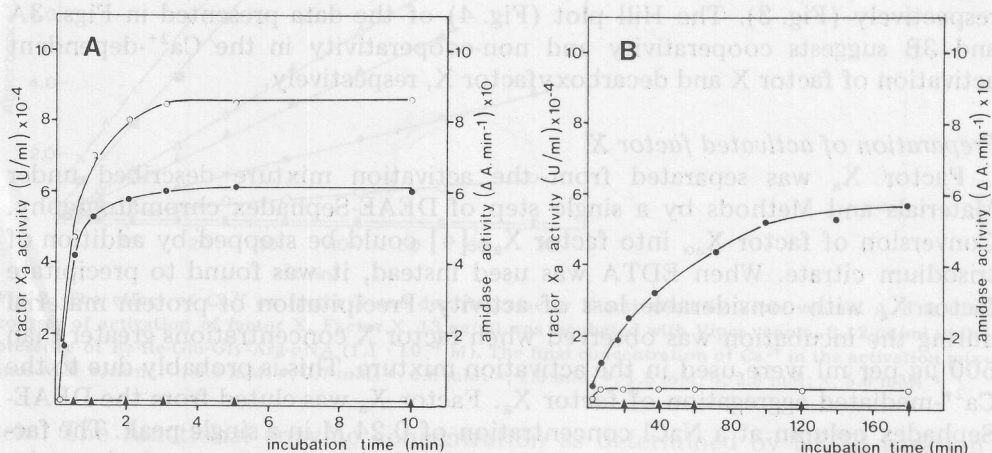


Fig. 1. Activation of factor X and decarboxyfactor X by Viper venom. A. Factor X (0.8 mg/ml) was incubated with Viper venom (10 µg/ml) and Ca²⁺, 10 mM final concentration. B. Decarboxyfactor X (1.0 mg/ml) was incubated with Viper venom (25 µg/ml) and Ca²⁺, 10 mM final concentration. ○—○, Factor X_a activity as determined by bioassay; ●—●, amidase activity towards Bz-Ile-Glu-Gly-Arg-pNA; ▲—▲, amidase activity, activation carried out in the presence of 10 mM EDTA.

factor X the protein band of the original protein decreases in intensity during the first minutes of activation. Parallel with the disappearance of this band a new, faster moving protein band appears. Appearance of the latter coincides with the generation of factor X_a and amidase activity (Fig. 1), and can be assigned to factor $X_{a\alpha}$ [4]. At longer incubation time a slightly faster moving protein band was also observed, this will be referred to as factor $X_{a\beta}$ [4]. Because the X_a activity and amidase activity remain unchanged, factors $X_{a\alpha}$ and $X_{a\beta}$ have the same specific activity.

Qualitatively, the same molecular changes as described for factor X were observed in decarboxyfactor X upon incubation with Viper venom. The appearance of a new faster moving protein band coincided with the generation of amidase activity. Both occurred at a slower rate with decarboxyfactor X than with factor X.

Influence of Ca^{2+} on rate of activation of factor X and decarboxyfactor X by Viper venom

The rate of factor X and decarboxyfactor X activation by Viper venom was measured by a continuous spectrophotometric assay as described under Materials and Methods. In the case of a linear rate of factor X_a or decarboxyfactor X_a formation, the increase of amidase activity towards Bz-Ile-Glu-Gly-Arg-pNA will be parabolic in time. A plot of absorbance versus t^2 yields a straight line, the slope of which indicates the initial velocity of the activation reaction [36]. The linear relationship between absorbance and t^2 obtained from activation experiments at variable Ca^{2+} concentrations is shown in Fig. 2.

The effect of Ca^{2+} on the initial rate of the activation of factor X and decarboxyfactor X by Viper venom is summarized in Fig. 3. It is shown that under optimal conditions the initial rate of factor X_a formation is about 60 times higher than the initial rate of decarboxyfactor X_a formation. Of particular interest in these studies were the differences in nature of the Ca^{2+} dependence: sigmoid and hyperbolic in case of factor X and decarboxyfactor X activation, respectively (Fig. 3). The Hill plot (Fig. 4) of the data presented in Figs. 3A and 3B suggests cooperativity and non-cooperativity in the Ca^{2+} -dependent activation of factor X and decarboxyfactor X, respectively.

Preparation of activated factor X

Factor X_a was separated from the activation mixture described under Materials and Methods by a single step of DEAE-Sephadex chromatography. Conversion of factor $X_{a\alpha}$ into factor $X_{a\beta}$ [4] could be stopped by addition of trisodium citrate. When EDTA was used instead, it was found to precipitate factor X_a with considerable loss of activity. Precipitation of protein material during the incubation was observed when factor X concentrations greater than 500 μ g per ml were used in the activation mixture. This is probably due to the Ca^{2+} -mediated aggregation of factor X_a . Factor X_a was eluted from the DEAE-Sephadex column at a NaCl concentration of 0.24 M in a single peak. The factor X_a prepared by this method can be practically homogeneous, as is shown by disc gel electrophoresis. It is further shown that factor X_a has a higher electrophoretic mobility than factor X (Fig. 5). The specific activity of the best preparations as determined by bioassay was found to be $3.0 \cdot 10^5$ U/mg pro-

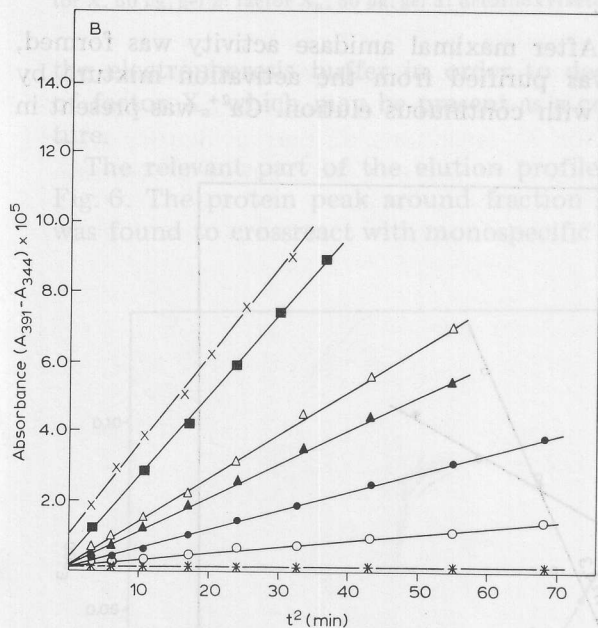
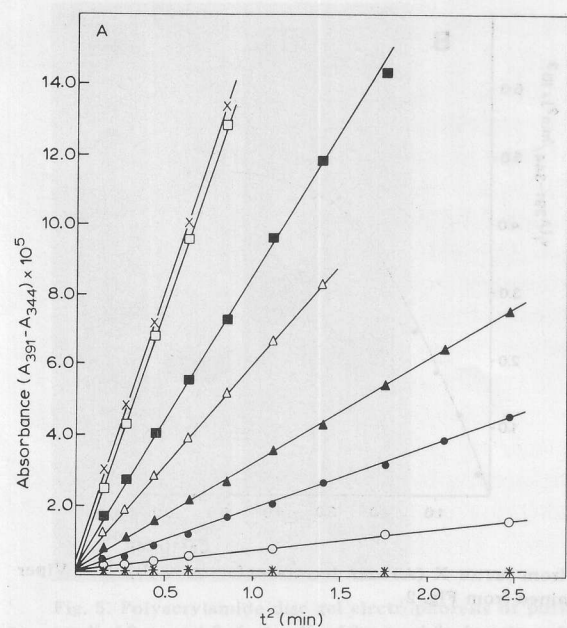


Fig. 2. The effect of Ca^{2+} on factor X and decarboxyfactor X activation by Viper venom. A. The time courses of activation of factor X. Factor X, $13 \mu\text{g/ml}$ was incubated with Viper venom, $0.12 \mu\text{g/ml}$ in the presence of Bz-Ile-Glu-Gly-Arg-pNA ($1.1 \cdot 10^{-4} \text{ M}$). The final concentration of Ca^{2+} in the activation mixture was: ★, none; ○, 0.6 mM; ●, 0.7 mM; ▲, 0.8 mM; △, 1.0 mM; ■, 1.5 mM; □, 2.8 mM; ×, 5.6 mM.

tein. The functional enzyme concentration as determined by NPGb titration was about 90% of the actual protein concentration ($5.5 \cdot 10^{-6} \text{ M}$).

Preparation of activated decarboxyfactor X

The activation procedure of decarboxyfactor X was performed as described

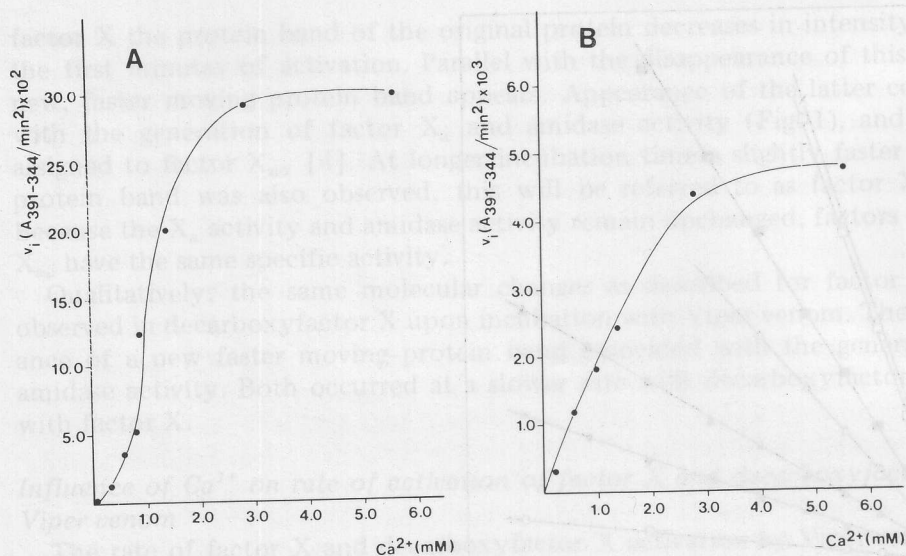


Fig. 3. Rate of generation of amidase activity from factor X (A) and decarboxyfactor X (B) with Viper venom at various Ca^{2+} concentrations, as determined from Fig. 2.

under Materials and Methods. After maximal amidase activity was formed, activated decarboxyfactor X was purified from the activation mixture by powder column electrophoresis with continuous elution. Ca^{2+} was present in

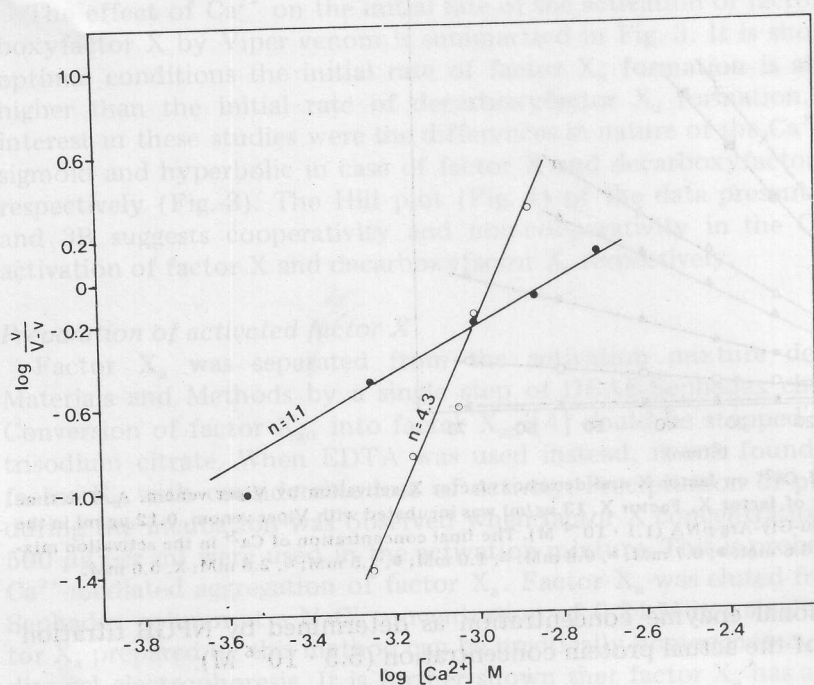


Fig. 4. Hill plot of the data presented in Fig. 3A and B, where v is the initial velocity at any Ca^{2+} concentration, V is the initial velocity at optimal Ca^{2+} concentration; open circles, factor X; closed circles, decarboxyfactor X.

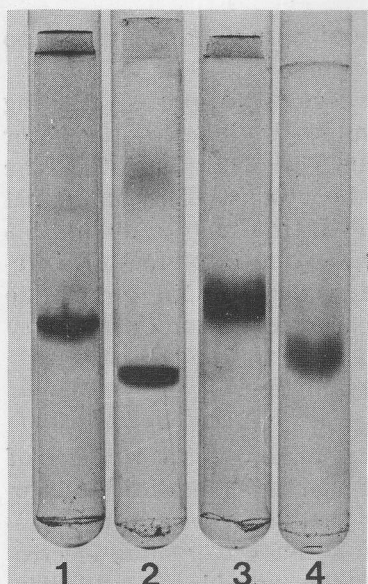


Fig. 5. Polyacrylamide disc gel electrophoresis of purified factor X_a and decarboxyfactor X_a . Gel 1: factor X, 50 μ g; gel 2: factor X_a , 50 μ g; gel 3: decarboxyfactor X, 50 μ g; gel 4: decarboxyfactor X_a , 30 μ g.

the electrophoresis buffer in order to decrease the electrophoretic mobility of factor X_a which may be present as a contamination in the activation mixture.

The relevant part of the elution profile of the electrophoresis is shown in Fig. 6. The protein peak around fraction 145 contained amidase activity and was found to crossreact with monospecific anti-bovine factor X antiserum. The

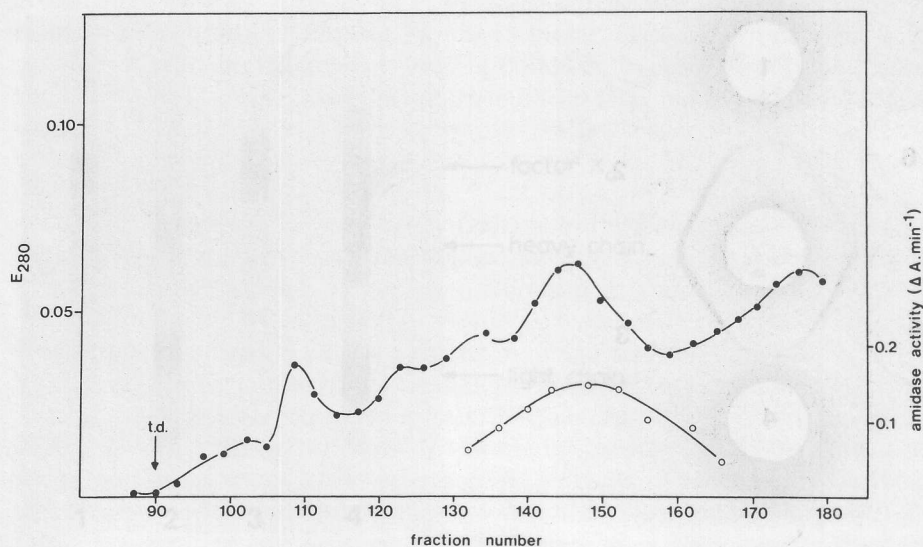


Fig. 6. Purification of activated decarboxyfactor X by preparative powder column electrophoresis. Experimental conditions as described under methods. ○, Protein (A_{280} nm), ●, amidase activity towards Bz-Ile-Glu-Gly-Arg-pNA; t.d., tracking dye (bromophenol blue).

decarboxyfactor X_a -containing fractions were pooled and concentrated to about 5 ml (Amicon PM-10 membrane).

The final yield is about 30%, as calculated from recovery of protein reacting with antifactor X. The specific activity was determined by bioassay for factor X_a activity was approximately $6 \cdot 10^2$ U/mg protein. This is less than 1% of the specific activity generally determined for purified factor X_a . The functional decarboxyfactor X_a concentration as assayed by NPGB titration was about 75% of the calculated protein concentration. The activated decarboxyfactor X prepared by this method in a good preparation is homogeneous in polyacrylamide gel electrophoresis and has, in absence of Ca^{2+} , an electrophoretic mobility equal to factor X_a (Fig. 5).

Zone electrophoresis of factor X_a and decarboxyfactor X_a

We performed an electrophoresis in agarose gel at pH 8.6 in the presence of EDTA or Ca^{2+} . Factor X_a and decarboxyfactor X_a have identical electrophoretic mobilities which are somewhat lower than that of factor X in the presence of EDTA. In the presence of Ca^{2+} , factor X_a has a very low electrophoretic mobility and even tends to migrate to the cathode. The electrophoretic mobility of decarboxyfactor X_a is retarded in the presence of Ca^{2+} .

Immunodiffusion

In double immunodiffusion experiments according to Ouchterlony [31] single precipitation lines were observed for anti-factor X against activated factor X and activated decarboxyfactor X. Reactions of partial identity were observed. The results indicate that the two proteins have antigenic determinants in common (Fig. 7).

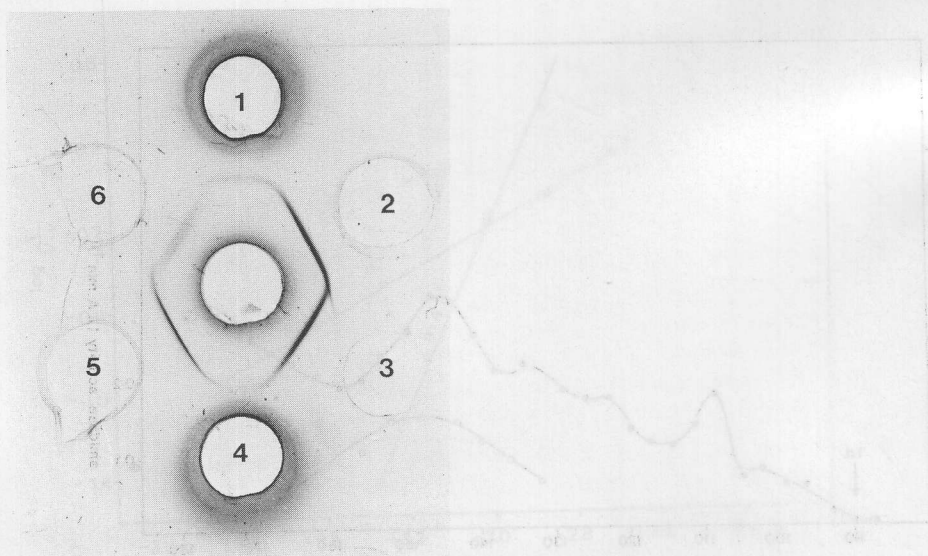


Fig. 7. Double immunodiffusion. Centre well contained anti-bovine factor X antiserum. Well 1, normal bovine plasma; well 2, factor X; well 3, factor X_a ; well 4, coumarin bovine plasma; well 5, decarboxy-factor X; well 6, decarboxyfactor X_a .

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate

Electrophoresis of purified factor X_a and decarboxyfactor X_a on polyacrylamide in the presence of sodium dodecyl sulphate according to Weber and Osborn [37] shows that both enzymes have an identical molecular weight of about 47 000 (Fig. 8, gels 1 and 3). Electrophoresis after disulfide reduction demonstrates that the proteins consist of two polypeptide chains with molecular weights around 36 000 and 18 000 (Fig. 8, gels 2 and 4). A comparison with reduced factor X or reduced decarboxyfactor X indicates that the molecular change by activation with Viper venom occurs by splitting off a peptide from the heavy chain. The molecular weight of the light chain remains unchanged in the activation process. Especially in less pure preparations, as used for this experiment, conversion of the α -form into the β -form of the (pro)enzyme can blur the bands.

Aminoterminal amino acid analysis

Aminoterminal amino acid analysis was performed with the method according to Gray [26]. For both factor X_a and decarboxyfactor X_a alanine and isoleucine were found as the aminoterminal amino acids. Earlier, alanine was reported as the aminoterminal amino acid of the light chain while tryptophane was probably the aminoterminal acid of the heavy chain of decarboxyfactor X [20]. It is obvious that after activation of decarboxyfactor X isoleucine becomes the new aminoterminal amino acid of the heavy chain, as is the case with factor X [13].

Amino acid analysis

The amino acid compositions for factor X_a and decarboxyfactor X_a are summarized in Table I. No significant differences in amino acid composition

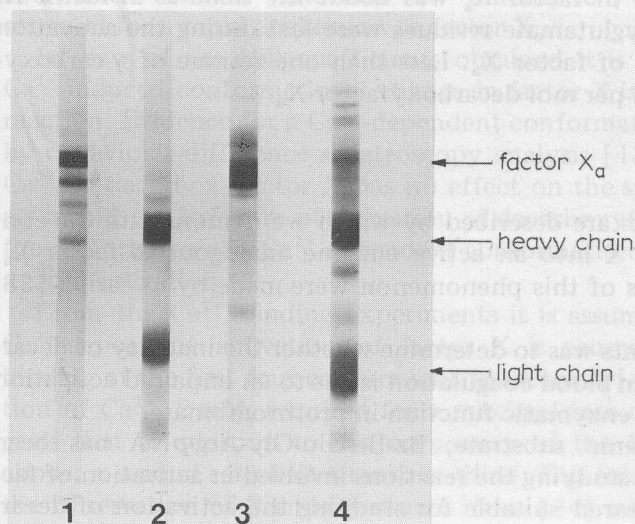


Fig. 8. Sodium dodecyl sulphate polyacrylamide gel electrophoresis. Gel 1, factor X_a , 15 μ g; gel 2, factor X_a , 30 μ g, disulfide reduced prior to electrophoresis; gel 3, decarboxyfactor X_a , 15 μ g; gel 4, decarboxyfactor X_a , 30 μ g, disulfide reduced prior to electrophoresis.

TABLE I

AMINO ACID COMPOSITION OF FACTOR X_a AND DECARBOXYFACTOR X_a

Amino acid	Factor X_a mol/47 000	Decarboxy- factor X_a mol/47 000	Factor $X_{a\alpha}$ *** $M_r=47\ 000$
Lysine	22	17	23
Histidine	8	8	10
Arginine	22	23	23
Aspartic acid	38	41	34
Threonine *	26	24	28
Serine *	25	27	24
Glutamic acid	53	58	52
Proline	13	12	15
Glycine	44	38	38
Alanine	28	25	26
Half-cystine	17	16	24
Valine	19	18	24
Methionine	6	6	5
Isoleucine	9	11	11
Leucine	26	26	23
Tyrosine	9	9	9
Phenylalanine	21	21	21
γ -Carboxyglutamic acid **	12	<1	—

* Values determined by extrapolation to zero time hydrolysis.

** Determined in an acid hydrolysate after reduction of the protein with [3H]diborane.

*** Determined from amino acid composition [21,22].

were observed. These amino acid analyses are in fairly good agreement with the data from the known amino acid composition [13,21,22].

γ -Carboxyglutamate residues were determined with a modified method according to Zytkevich [27] as described in ref. 20. The number of γ -carboxyglutamate residues detected in factor X_a was about the same as in factor X, indicating that no γ -carboxyglutamate residues were lost during the activation and subsequent purification of factor X_a . Less than one residue of γ -carboxyglutamate residue was found per mol decarboxyfactor X_a .

Discussion

In this article experiments are described by which we demonstrate the conversion of decarboxyfactor X into an active enzyme analogous to factor X_a . Preliminary communications of this phenomenon were made by us earlier [38, 39].

The aim of the experiments was to determine whether the inability of decarboxyfactor X to participate in blood coagulation is due to an impaired activation mechanism and/or impaired enzymatic function in prothrombinase.

The synthetic chromogenic substrate, Bz-Ile-Glu-Gly-Arg-pNA has been shown to be a useful tool in studying the reactions involved in activation of factor X [40,41]; it also appeared suitable for studying the activation of decarboxyfactor X.

In contrast to activation by the physiological activators, factor VII $_a$ and factor IX $_a$, the activation of factor X by Viper venom does not require phos-

pholipids [4]. However, the mechanism of activation of factor X by physiological and non-physiological activators has been shown to be identical [4].

Under the conditions used, factor X was completely activated within three minutes and clotting and amidase activity increased in parallel (Fig. 1a). Under the same conditions the complete activation of decarboxyfactor X, as measured by amidase activity, requires about 180 min (Fig. 1B).

After that time about the same amidase activity as in the factor X activation was reached. The possibility of converting decarboxyfactor X in an enzyme analogous to factor X_a was also confirmed by the comparable time course of generation of amidase and the limited proteolysis as visualized by SDS gel electrophoresis. The time course of generation of clotting activity is qualitatively and quantitatively quite different in the two preparations. Therefore, it is concluded that no clotting activity can be generated from decarboxyfactor X. The small amount of factor X_a activity generated from decarboxyfactor X must be due either to a contamination by factor X and/or to a small endogenous factor X activity of decarboxyfactor X_a (see below).

To our surprise the activation of decarboxyfactor X depends on the presence of Ca^{2+} . This Ca^{2+} requirement is unexpected because it is known that the factor X activating fraction of Viper venom binds no Ca^{2+} [42], and decarboxyfactor X has a very low affinity for Ca^{2+} , due to the absence of γ -carboxyglutamate residues [43]. It was shown that the maximal rate of factor X activation is about 60 times higher than of decarboxyfactor X, whereas the velocity of activation of factor X is dependent upon Ca^{2+} concentration in a sigmoidal fashion; that of decarboxyfactor X is hyperbolic.

A Hill plot of the data obtained indicates that only in the case of factor X the increase in Ca^{2+} concentration has a positive cooperative effect on the reaction velocity ($n_H = 4.3$).

We conclude that the substrate for Russell's Viper venom-X is the protein complexed with Ca^{2+} and that Ca^{2+} binding to γ -carboxyglutamate residues is essential for a rapid conversion to factor X_a .

In view of the Hill coefficients obtained it is tempting to speculate that a Ca^{2+} -induced conformational change in factor X is important for the activation reaction. Evidence for a Ca^{2+} -dependent conformation in factor X was obtained by ultraviolet difference spectroscopy analysis [43]. The fact that addition of Ca^{2+} to decarboxyfactor X has no effect on the spectra of the aromatic amino acids and the low rate of activation of decarboxyfactor X clearly demonstrates the importance of a Ca^{2+} -dependent conformational change in the substrate for a rapid activation.

From the Ca^{2+} binding experiments it is assumed that cooperativity in the Ca^{2+} -dependent activation of factor X is caused by a positive cooperative binding of the first four calcium ions to factor X [43]. However, the concentration of Ca^{2+} , 2 mM, required for a maximal rate of activation of factor X suggests that in addition to the occupancy of the Ca^{2+} binding sites mentioned above, a second set of sites must be filled. This interpretation is consistent with the observation of Prendergast and Mann [44] who showed that the maximal rate of activation of prothrombin is found at a Ca^{2+} concentration which greatly exceeds that required to fill the Ca^{2+} binding sites which exhibit positive cooperativity.

Separation of activated factor X from the activation mixture could be achieved by DEAE-Sephadex chromatography as described by Jesty [45]. Using this procedure we did not succeed in separating activated decarboxyfactor X from its activation mixture. Using powder column electrophoresis a homogeneous preparation, as judged by polyacrylamide electrophoresis, could be obtained. Addition of Ca^{2+} to the electrophoresis buffer was carried out in order to obtain a separation between decarboxyfactor X_a and contaminating factor X_a . In spite of this, a residual factor X_a activity (about $6 \cdot 10^2$ U/mg protein) can be detected in the purified decarboxyfactor X_a preparation. The presence of factor X_a activity in the purified product may be due to a persistent contamination or to an intrinsic activity of decarboxyfactor X_a , as in the case of decarboxyfactor II [46]. Differentiation will only be possible when high yields of decarboxyfactor X_a enable us to carry out rigorous tests for purity of this product.

As measured by sodium dodecyl sulphate gel electrophoresis, factor X_a and decarboxyfactor X_a have equal molecular weights. Fig. 8 suggests that two forms of the enzymes may exist with molecular weights of approximately 47 000 and 43 000. Upon reduction prior to electrophoresis it is shown that the heterogeneity is due to a difference in molecular weight of the heavy chain. Because the only aminoterminal amino acids found were isoleucine and alanine, the difference must be caused by splitting off a peptide at the carboxyterminal end of the heavy chain. These results are consistent with the two forms of factor X_a reported by Fujikawa [14] and Jesty [47]. Further proof for the conversion of decarboxyfactor X into an active enzyme analogous to factor X_a was obtained by an immunological technique and amino acid analysis. It has been shown that decarboxyfactor X_a and factor X_a have common antigenic determinants (Fig. 7). Important differences in amino acid composition were only found with respect to γ -carboxyglutamate content (Table I). Moreover, the γ -carboxyglutamate content of factor X_a is unaltered as compared with factor X. This result indicates that all γ -carboxyglutamate residues are conserved after activation by Viper venom. A striking difference of electrophoretic mobility of factor X_a and factor X was found in the presence of Ca^{2+} . The most likely explanation for this phenomenon might be the tendency of factor X_a to aggregate in the presence of Ca^{2+} [45]. Also the electrophoretic mobility of decarboxyfactor X_a , in the presence of Ca^{2+} , is somewhat decreased. Whether this can be due to aggregation is not yet clear.

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